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Understanding the relationship between norovirus and indicator organisms on predicted contamination of produce along the United States-Mexico border

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B.S.
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Abstract

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By Alexandra G. Wickson

Previous research has shown that norovirus is the primary cause of foodborne outbreaks in the United States and that of foodborne illnesses caused by viruses in particular, norovirus accounts for almost all cases of illnesses. Common sources of food contamination are grower's hands, produce contamination in the field, and hands of workers preparing food for the consumer.

Norovirus is preventable, but challenges include: viral persistence on surfaces, low concentrations in samples, and lack of field and cost friendly detection methods. Typically, indicator organisms, which are microbes that are present in fecal contamination and easier to detect, are used to decide if pathogen presence in a sample is likely. Research to understand the relationship between presence of bacterial and viral indicators and presence of norovirus on different environmental samples is needed. This study aims to determine if bacterial and viral indicators can inform the presence of norovirus on grower's hands, produce, and in water of crops grown along the US-Mexico border.

From 2011-2012, hand-rinse, soil, water (source and irrigation), and produce (melons, jalapeños, tomatoes) samples were taken from 11 farms. Each sample was assayed for indicators: *E. coli*, coliforms, *Enterococcus*, *Bacteroidales*, and coliphages. Intra-indicator relationships were evaluated by sample type using a Spearman rank-correlation test. Norovirus presence was examined by testing a subset of 50 samples and reporting proportions of samples positive for either norovirus genotype GI or GII. Logistic models and odds ratios were calculated to determine if there was a relationship between indicator presence and norovirus presence (α=0.05).

When *E. coli* was present on produce, there was a significant relationship with coliforms (OR=24.54; p=0.0285), *Enterococcus* (OR=2.84; p=0.0012), and coliphages (OR=2.34; p=<0.0001). No other indicators had significant relationships with other indicators. By sample type, *Enterococcus* showed strong, significant relationships with coliphages (rho=0.6242) and coliforms (rho=0.6251) on produce samples. Norovirus was present on 6/50 (12%) samples, five of which were positive for genotype II. Of the samples positive for norovirus, 50% were from source water, 33% from hand-rinses, and 17% from produce. There was no relationship between norovirus presence and any of the indicators tested. Overall, the low proportion of samples positive for norovirus and the absence of a relationship between norovirus and any indicator implies that these indicators are not a good predictor of norovirus contamination.

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Literature Review

I. Produce Contamination and Pathogens associated with Outbreaks

In the United States, major pathogens cause over nine million foodborne illnesses every year (1). A study estimating major foodborne pathogens in the United States revealed that the majority of foodborne illnesses (59%) are caused by viruses, with bacterial (39%) and parasitic (2%) causes being less common (2). While most foodborne illnesses are caused by viruses, the majority of hospitalizations (64%) and deaths (64%) related to foodborne outbreaks are caused by bacterial pathogens (2). The bacteria that cause the most illness are *Salmonella*, *Clostridium perfringens*, and *Campylobacter,* with those three bacteria combined causing 30% of all foodborne illnesses (2). *Salmonella* leads all pathogens for the most hospitalizations of all foodborne illness (35%) and the most deaths (28%) (2). Meanwhile, the top parasitic diseases attributable to foodborne illness are *Giardia*, *Cryptosporidium*, *Cyclospora*, and *Toxoplasma* (2). About 30% of foodborne illnesses due to produce specifically are caused by parasites, which is comparable to the percentage of foodborne illnesses due to produce caused by bacteria (27%) (1). The leading pathogens responsible for illness, hospitalization, and death are all bacterial or parasitic, with one exception: norovirus (2).

While bacterial pathogens cause more hospitalizations and deaths than viruses, viral pathogens (i.e. norovirus, hepatitis A) are responsible for 59% of foodborne illnesses, 27% of hospitalizations, and 12% of deaths (2). In a review of foodborne outbreaks from 1998-2008, norovirus caused 57% of all illnesses, regardless of food item (1). Additionally, of the 59% of foodborne illnesses that viral pathogens cause, norovirus was responsible for almost all (58%) of them (2). Norovirus is the second leading cause of hospitalization (26%) and the fourth leading cause of death (11%) due to foodborne illness (2). When reviewing outbreaks specific to produce contamination, norovirus becomes even more important. Almost half of all foodborne illnesses

can be attributed to contaminated produce and of these illnesses, over 60% are caused by viruses, largely due to norovirus (1). Produce was suspected in over half of norovirus outbreaks from 2001-2008 that were attributed to a single food item (3). Clearly norovirus is an important contributor to foodborne illnesses, especially those where produce is suspected.

II. Norovirus

The characteristics of norovirus itself, the presentation of symptoms and people affected, as well as the exposure pathways are all factors that contribute to the contamination and spread of norovirus as a foodborne illness. Norovirus is highly contagious and causes gastroenteritis, vomiting, and diarrhea (4). A member of the *Caliciviridae* family, norovirus is also divided into six genogroups and 31 genotypes (5, 6). The most common cause of human illness is due to noroviruses in genogroup II, genotype 4 (5). Only a few viral particles, as few as 20, are needed to cause illness and illness can be acquired from simple proximity to a sick person that has vomited (5). Proximity to another sick person is not the only way norovirus can be acquired. People can also get norovirus from contaminated surfaces, food, and water. In fact, norovirus is the primary cause of foodborne outbreaks in the United States. It causes at least 20 million illnesses every year, yet this is likely an underestimate because norovirus is commonly not reported (4, 6). Symptoms attributed to norovirus are typically mild, but global burden is high (7). Norovirus accounts for 18% of gastroenteritis cases globally (8). The majority of norovirus illnesses in the U.S. are related to foodborne outbreaks and the majority of foodborne outbreaks are caused by infected food workers (4). The foodborne outbreaks of norovirus due to infected food workers are preventable. Over half of norovirus outbreaks caused by infected food workers are due to handling food without gloves, and about 20% of food workers report coming to work while sick because they fear repercussions (4). This food preparation step of the food production

chain is the easiest to observe and pinpoint paths of potential contamination, but to thoroughly address foodborne illness, we need to study all steps of the production chain.

III. How produce becomes contaminated with pathogens

Understanding how produce gets contaminated and how contamination persists throughout the production chain is crucial to being able to detect and prevent contamination. Pathways for contamination include fecal microbes from wild or domestic animals infiltrating soil, composting material, or water used for irrigation (9). The magnitude of this contamination depends on the type of produce and environmental factors such as rainfall and land use around the farm (9). Additionally, worker hands are a source of contamination, often due to poor worker hygiene. A USDA report showed that of eight categories of produce, all were mostly or partially harvested by hand (10). Workers that harvest this produce by hand often do not have access to running water or soap and hand sanitizer is not as effective as traditional soap (11), so poor worker hygiene during harvesting and processing can lead to contamination on produce.

When produce leaves the field, it is transported to large processing or packing facilities. Here, contaminated surfaces or the quality of water used for washing produce can have a large impact on the microbial load of the produce (12). In one study, a stainless-steel surface inoculated with norovirus still had detectable norovirus RNA after 70 days (13). Water used for washing can even increase cross-contamination and microbial loads (12). In a study looking at packing sheds, 24% of equipment samples tested positive for *Salmonella* and 83% of those that tested positive were from surfaces that contact the final, washed product (14).

From the processing facility, produce is distributed, allowing for surface contamination or cross-contamination with other produce (15). Norovirus, for example, can survive on surfaces for weeks and the virus persists on produce at refrigeration temperatures (16). The time it takes for

produce to be shipped across countries, reach consumers, and result in an outbreak, makes it difficult to trace contamination to one source. Understanding sources of contamination is a major step in detecting and preventing foodborne illness and outbreaks.

IV. Produce Safety

The burden of foodborne illness and the challenge of preventing contamination in our food production system necessitates interventions to decrease the risk of foodborne illness. Some interventions have been studied, though none are ideal. On a broad level, policy affects change in food safety. To address food safety concerns of imported produce, the U.S. established the Food Safety Modernization Act (FSMA) in 2011 that allows the Food and Drug Administration (FDA) to establish additional standards that imported produce must meet (17). In the FSMA's mandatory rules, facilities that deal with food must develop plans for prevention of contamination and comply with new produce safety regulations (18). Additionally, the act establishes rules that address inspection, response for outbreaks, import regulations, and the FDA's domestic and foreign partnerships (18). The FSMA's Produce Rule sets standards for fruit and vegetables throughout the food production process (19). The Produce Rule addresses agricultural water and soil quality, access of animals to produce, worker hygiene, and equipment sanitation (19).

On a smaller scale, individual farms and production facilities can implement more specific interventions. At the production level, interventions are focused on improving quality of water used for irrigation or worker hand hygiene (7). Handwashing with soap and water, while widely known to be effective at reducing microbial load on hands, has not been studied in agricultural settings as it relates to produce contamination. Additionally, hand hygiene in agricultural settings is complicated by the fact that facilities are sometimes inaccessible or not provided (20).

Alcohol-based hand sanitizers (ABHS) have been investigated as a possible alternative to hand washing when soap and water is not available and ABHS is also effective at reducing fecal contamination (20).

In the processing facilities, water quality is the biggest concern. Sanitizers are recommended for maintaining quality of the water used to wash produce (12). Additionally, factors affecting produce contamination can include: application method of the decontamination treatment, number of times the produce is washed, whether different produce is washed together, and the concentration and contact time of sanitizers (12).

When food reaches the consumer, there have been many opportunities for contamination, but handling and preparing food properly reduces the risk of foodborne illness. Preventing cross-contamination of uncooked meat, cooking food to proper temperatures, and ensuring good worker hygiene through handwashing and keeping sick workers at home are ways to prevent contamination at the preparation stage (4).

V. Norovirus and Produce Contamination

Research into norovirus-specific interventions for reducing contamination and transmission of norovirus is lacking, but it can be prevented through handwashing, thorough disinfection, proper cooking temperatures, and avoiding food preparation and other people when sick (4). However, there are still many challenges. Challenges in preventing illness caused by norovirus include the small number of viral particles required to cause illness, the highly contagious nature of the virus, the capability of the virus to remain on surfaces for weeks, and the virus's ability to remain infectious after freezing, heating, and disinfection (4). While norovirus typically resolves without treatment within a few days, the infective virus can still be shed for weeks, increasing the risk for contamination of produce by workers that are unaware

they are still shedding infectious particles (6). The burden of illness and challenges in prevention necessitate further research into routes of transmission for norovirus.

There are many routes of transmission for norovirus, from contamination in the field to contamination in a restaurant or home. Norovirus is present on produce in low concentrations, making it especially difficult to detect and prevent (21, 22). At the handling and processing stages, there is no obvious guidance for norovirus prevention. Contamination may occur through handling of produce during harvest or from water of poor quality used in irrigation or washing, which only underscores the importance of proper handwashing for farm workers and improving quality of agricultural water as prevention methods (23). While norovirus contamination can occur in these early steps of the food production chain, the primary source of norovirus contamination on produce is transmission from worker hands in restaurants (24). Food workers in restaurants cause about 70% of foodborne norovirus outbreaks (4). Norovirus spreads readily and food workers increase the risk by coming to work sick, washing their hands improperly, or touching prepared food with their bare hands (4). While contamination from food workers is a likely source of a norovirus outbreak, it is still important to address norovirus contamination at all levels of food production.

VI. Evaluating Norovirus Contamination

Evaluating norovirus contamination is challenging for four reasons. The first is that detecting and testing for viral contamination on produce and in the environment is difficult due to the complexity of preparing the sample for molecular amplification (6). The sample must first be eluted and concentrated before RNA extraction and molecular amplification can be done (6). Secondly, since this detection method relies on nucleic acid detection, it is impossible to distinguish between infectious and non-infectious virus (5). This complicates studies that utilize

this method to investigate produce contamination and foodborne illness given that the detected viral particles may not be infectious (5). Next, until recently, there was no cell culture system for norovirus, meaning that it was impossible to determine viability and infectivity of the virus in the sample (6). The culture system that is now newly developed uses derived stem cells to mimic the human intestine, allowing norovirus to replicate (25). While this new culture system provides benefits, not all genotypes replicate equally as well and the culture system is expensive and challenging to work with (25). Finally, both the culture system and molecular amplification for norovirus are not field friendly and can require resources that some local laboratories and countries may not have access to. In general, viral detection methods are not standard across laboratories, so it would be time consuming to receive results, which would make required or routine testing of norovirus challenging and impractical (7). Because norovirus contamination is difficult to measure, microbial indicator organisms that are present in fecal contamination (such as *E. coli*, *Enterococcus*, coliforms, coliphages, or *Bacteroidales*), are used to try and inform us about the presence of norovirus since the indicator microbes are easier to measure.

VII. Indicator Organisms

Viruses are difficult to detect in agricultural environments; there is an abundance of potential pathogens, and detection has high costs and large sample size requirements (26). For these reasons, indicator organisms that are easier to detect are used to indicate fecal contamination, which could inform the presence of viral pathogens that are passed through feces (26). In the FDA's Produce Safety Rule, testing for microbial indicators is required as a method of determining contamination potential in water used for agriculture (26). In recreational water, bacterial and protozoan pathogens are more likely to show a relationship with indicator bacteria than viral pathogens are (27). The relationship between alternative indicators and pathogens has

not been supported (27). Despite this, because measuring pathogens themselves can be difficult, indicator organisms are still used. Since indicator organisms are used to predict potential pathogen presence, it is crucial to understand the relationship between indicator and pathogen presence. *E. coli*, *Enterococcus*, and coliforms are typically used as indicator organisms, though *Bacteroides* spp. and viral coliphages can be used as well (28). The common bacterial indicators (*E. coli*, coliforms, and *Enterococcus*) all measure fecal contamination (28). However, *E. coli* is present in both human and animal feces, coliforms are present in wastewater, and *Enterococcus* can come from non-fecal sources as well so the source of contamination may not be identifiable (28). *Bacteroides* spp. are a common fecal bacteria but their correlation with pathogens has not been established (28). Viral coliphages present a similar problem as the bacterial indicators, as they are present in human feces, animal feces, and sewage, complicating identification of the contamination source (28). The benefit of coliphages is that they could be a better indicator for viral pathogens as opposed to bacterial indicators because coliphages are viruses and therefore may be more similar to viral pathogens than bacterial indicators would be (28). Meanwhile, some pathogens that are difficult to detect are human-only pathogens, such as norovirus. This means that even if samples are positive for both the pathogen and the indicator organism, it cannot be concluded the contamination is from a human source. Studies looking at the relationship between indicator organisms and norovirus contamination on produce are lacking. However, another study showed that coliphages may be a more accurate predictor of norovirus than indicator bacteria, so there is still work to be done on organisms used for predicting viral contamination (29).

The best indicator measure would be easy to detect and in measurable concentrations, but the best indicator organism often depends on the sample type. Some of the most commonly used

indicator organisms are *E. coli, Enterococcus*, and coliforms, but alternative indicators, such as coliphages are becoming more accepted (30). If testing produce, indicator organism concentrations can vary according to how the produce is grown and harvested, and the packing process (31). In summary, given the large percentages of produce that are harvested by hand and the burden of foodborne illness, it is important to further our understanding of how indicator detection can predict pathogen presence, especially norovirus.

VIII. US-Mexico Border

Improving our understanding of norovirus on produce and how to measure it, is especially important along the border of the United States and Mexico. One of the United States' largest trading partners is Mexico, with Mexico making up about 70% of fresh vegetables imported into the U.S., and about 30% of fruit imports (32). The large amount of produce coming from this region, along with the added challenges of varying regulations between the countries and differing abilities to test for norovirus, highlights the need to study norovirus's relationship with indicator organisms in this area in particular. Not surprisingly, the large amount of produce coming from Mexico also means produce originating in Mexico accounts for a majority of foodborne outbreak sources (33). From 1996-2014, there were 42 outbreaks with Mexican produce as the suspected source, while Indonesia, the country suspected second-most, only accounted for 17 outbreaks (33). Mexico's Service for Agroalimentary Public Health, Safety, and Quality (SENASICA) and Federal Commission for the Protection from Sanitary Risks (COFEPRIS) have partnered with the U.S. FDA to fortify food safety measures (17). A recent example is SENASICA's establishment of the System for Risk Reduction from Contamination (SRRC), which aims to prevent contamination in the early produce production stages (17). Though Mexico is taking appropriate steps, there are still challenges in the country's

infrastructure and practices that need to be addressed. There is little research on these challenges, but factors considered in previous foodborne outbreaks include the quality of water used for irrigation or handwashing, unclean transport conditions for the produce, and lack of appropriate sanitary facilities for workers (34). Additionally, Mexico must comply with the FSMA Produce Rule, which established new standards for the production, packing, and transporting of produce that will enter the U.S. (17). Given the new regulatory requirements, other challenges Mexico faces and the reliance of the U.S. on Mexico for a large percentage of imported produce, the U.S.-Mexico border is a key region to study.

IX. Need, Goal, Aims

In order to reduce the burden of foodborne illness due to contamination of produce grown along the US-Mexico border, there is a need to understand the relationship between presence of bacterial and viral indicators and presence of norovirus on grower's hands, produce, and in water of crops grown along the US-Mexico border. The goal is to determine if bacterial and viral indicators can inform the presence of norovirus on grower's hands, produce, and in water of crops grown along the US-Mexico border. This goal is accomplished through three aims. Aim 1 is to assess the presence, quantity, and intra-indicator relationship of bacterial and viral indicators (such as *E. coli, Enterococcus*, coliforms, *Bacteroidales*, etc) by sample type grown along the US-Mexico border. Aim 2 is to assess the presence and quantity of norovirus by sample type and produce type grown along the US-Mexico border. Aim 3 is to determine if bacterial and viral indicator presence and quantity has a relationship with norovirus presence and quantity.

X. Significance

Norovirus is responsible for a majority of foodborne outbreaks and a high burden of morbidity. Reducing norovirus transmission involves interventions at each step of the food production chain. At the production and processing stages, pinpointing possible sources of contamination requires detection of norovirus on produce and environmental samples. While detecting norovirus in these samples is possible, it is challenging, time consuming, and expensive, so alternatives are needed. Previous research on using indicator organisms to detect fecal contamination could be considered as a proxy for norovirus contamination, allowing for prediction of possible norovirus exposure points. The ability to pinpoint where norovirus exposure is happening in the food production chain can inform regulation and hygiene interventions to reduce contamination. Minimizing norovirus illness will impact health, economics, and substantially reduce foodborne outbreaks overall.

Data and Methods

Study Sites

This study is part of a larger study researching fecally-associated pathogen contamination in produce collected from farms in three northeastern states of Mexico along the U.S. border (Nuevo Leon, Coahuila, and Tamaulipas) from 2011-2012 (31, 35). These states were chosen due to their volume of target produce, ease of sampling logistics, and high export volume of crops to the United States. Farms identified by Mexican state produce associations and the state Secretariat for Agriculture were selected to include 3-5 farms per produce item (melons, tomatoes, and jalapeños) and were consented before sample collection. Of the 11 farms enrolled, four grew both tomatoes and jalapeños, one grew jalapeños exclusively, one grew tomatoes exclusively, and five grew melons (cantaloupes). Institutional Review Boards (IRB) at La Universidad Autónoma de Nuevo León (UANL), North Carolina State University (NCSU), and Emory University (Emory IRB: 00035460, Appendix A) reviewed and approved the protocol. Data access privileges were granted by Juan Leon at Emory University. When referring to the data in this study, produce can refer to a category to which all samples are associated with or can refer to the variable "produce type" which represents individual produce items. To eliminate confusion, this paper will use the term "produce" to refer to produce generally and as a group that includes both individual produce items and produce-associated samples (ex. Hand-rinses, water samples). When a sample may be related to produce, such as a hand-rinse sample being labeled as from the hand of a grower that touched tomato or melon, terms will incorporate the specific produce item and the sample type (ex. tomato-associated hand-rinse or melon-associated soil). When referring to a produce sample that is the produce itself, the specific produce term (melon, tomato, jalapeño) will be used.

Study Design

Sample Collection

Sample collection was done in accordance with protocols used in Bartz et al. 2017 (35). Samples were taken throughout the production process: before and after harvest, during distribution, and at the packing shed. Produce rinse samples (n=243, 1500mL) were a composite of three subsamples and were collected in 0.1% peptone water from jalapeños (n=152), tomatoes (n=199), and melons (n=266). Matched hand rinses (n=168, 2250mL) from workers were also collected in 0.1% peptone water and were taken directly after harvesting of produce. Soil samples (n=85, 225mL) were collected from near the produce immediately before harvest. Water samples included both source water (n=48, 4500mL) from source well pumps and irrigation water samples (n=73, 4500mL) from irrigation hoses, collected after disinfection of the hose and allowing the hose to run for 30 seconds.

Microbial Indicator Testing

Microbial indicator testing was done in accordance with protocols used in Heredia et al. 2016 (31). Each composite sample was tested at UANL within 24 hours of collection for three bacterial indicators (general *E. coli*, coliforms, *Enterococcus*) and one viral indicator (somatic coliphages). Bacterial indicators were assessed by first counting the number of colony forming units (CFU) that grew on selective media for each replicate sample. These collective counts were combined with sample volumes to get an overall measure of indicator growth, according to U.S. Food and Drug Administration (FDA) protocols (36). If growth was observed on any plate, the sample was positive for the indicator. Coliphages were assayed using the Fast Phage most probable number (MPN) Quanti-Tray method (Charm Sciences, Lawrence, MA) and the MPN was calculated using an IDEXX Quanti-Tray/2000 MPN table (IDEXX Laboratories, Westbrook, ME) (37). If a UV light detected fluorescence, the sample was positive.

Norovirus Testing

Frozen rinsate samples and agricultural water samples were shipped to Emory University for norovirus presence testing. The samples were first eluted using a protocol adapted from Pickering et al., 2012 (38) that uses alkaline pH to separate any norovirus particles from soil particles. Adjustments made to the Pickering protocol included increases in pH to 9 and in NaCl concentration to 0.15M. After the sample settled, the solution above the precipitate was used for analyses. Primary concentration methods were modified from the Guevremont et al. 2006 study (39). Modifications made were addition of reagents [0.15M NaCl (total concentration 0.3M NaCl), 12% weight/volume Polyethylene Glycol 8000 (PEG 8000; Spectrum; New Brunswick, NJ), and 1% weight/volume Bovine Serum Albumin (BSA; Calbiotech; Spring Valley, CA)], a pH adjustment to 7-7.5, and overnight incubation of samples at 4°C followed by centrifugation for 30 minutes at 4°C and 12,000 RPM. After centrifugation, the supernatant was discarded and the pellet resuspended in 3mL TBS with Tween-20X (Fisher Scientific, Waltham, MA).

For the secondary concentration, the resuspended pellet was further concentrated using an additional 12% weight/volume PEG 8000. The sample was then incubated at 4°C on a rotator for 1 hour and centrifuged at room temperature for 15 minutes at 10,000 RPM. The pellet was then resuspended in 100ul nuclease-free water.

RNA extraction was performed with a QIAamp Viral RNA Mini Kit (Qiagen; Germantown, MD). The only adjustment made to the viral extraction kit's standard protocol was to centrifuge the samples for three minutes at 7,000 RPM before adding the supernatant to the filter. Real-time PCR for GI and GII was performed following an adapted protocol from Kageyama et al. 2003 (40). The adapted protocol used 40 cycles, denaturing for 15 seconds at 95°C, then annealing and extending for 1 minute at 60°C. An internal amplification control (IAC), was utilized to reduce false negatives by identifying PCR inhibition (41). Samples were

run in duplicate both with and without IAC and if inhibition occurred, the test was repeated at a 1:4 dilution (41). A cycle-threshold count (C_t) of < 40 was used as a threshold for samples presumed to be positive. All PCR experiments included a positive (C_t = 27-29), negative (C_t > 40) and IAC control (C_t = 29-31) run in duplicate and samples were repeated if any controls did not amplify within their C_t limits.

Statistical Analyses

All statistical analyses were completed using SAS 9.4 (SAS Institute Inc., Cary, NC). Confidence intervals and p-values were used to assess statistical significance with a p-value of 0.05 or less considered to be significant. Data quality checks were performed to ensure data validity for all three datasets before merging them. For the indicator dataset, the arithmetic mean of the $\log_{10}$-transformed variables was calculated and normality of each indicator variable assessed. For the *Bacteroidales* dataset, the arithmetic mean, minimum, and maximum were calculated for the $\log_{10}$-transformed variable. The proportions of positive samples were also calculated for the entire sample and stratified by sample type. These values were all compared to values obtained in Ravaliya et al. 2014 which used the same data (42). Though there was a slight discrepancy in sample size (our dataset N=168, published dataset N=174), the quality check values were all still comparable considering the difference in sample size. For the norovirus dataset, all data were reviewed manually since there were only 50 samples. Datasets were merged using the following variables: New_Sample_ID for the indicator dataset, Emory_Sample_Code for the *Bacteroidales* dataset, and New_Sample_Code for the norovirus dataset.

While normality could not be confirmed using common normality tests (Shapiro-Wilks and Kolmogorov-Smirnov), the $\log_{10}$-transformed variables showed skewness, kurtosis, mean, and median values indicative of normal distributions. Furthermore, the graphs of the $\log_{10}$-

transformed variables were less skewed when they were not transformed or transformed using alternative methods. Based on these results, the $\log_{10}$-transformed variables were used for analysis despite the Shapiro-Wilks and Kolmogorov Smirnov tests for normality not reflecting a normal distribution.

Descriptive statistics were calculated for each $\log_{10}$-transformed indicator concentration, including mean, range, and standard error. For each indicator, the proportion of positive samples was calculated for the entire sample, as well as stratified by sample type. To assess concentration of indicators on samples, the arithmetic mean was calculated for the $\log_{10}$-transformed variables. To test intra-indicator relationships, Spearman rank-correlation values were calculated by sample type. Discussion of these correlation measures later will use the definition from the British Medical Journal regarding correlation strength: 0-0.19 is very weak, 0.2-0.39 is weak, 0.40-0.59 is moderate, 0.6-0.79 is strong and 0.8-1 is very strong (43).To assess the relationship between indicators based on presence and to assess the relationship between norovirus presence and indicator presence, logistic models were used to calculate odds ratios and 95% Wald confidence intervals. To determine the strength of the relationship between indicator presence and norovirus presence, an odds ratio was also calculated for all bacterial indicators as a whole. In models where cells had zero values, a Firth correction was used.

Results

Descriptive Statistics

Samples were tested for five indicator microbes: coliforms (N=622), *E. coli* (N=637), *Enterococcus* (N=633), coliphages (N=459), and *Bacteroidales* (N=168). Fifty samples were tested for norovirus including: 18 hand-rinse samples, 5 irrigation water samples, 8 source water samples, 17 produce samples, and two samples that were missing data on sample type. Of 638 samples taken from 11 farms, the majority of samples were from produce (N=243) and were taken before harvest (N=296). Produce-associated sample sizes were similar among jalapeños (N=152), melons (N=266), and tomatoes (N=199).

Assessing the presence, quantity, and relationship of indicators

Proportions of samples positive for each indicator were calculated to assess indicator presence for the entire sample and stratified by sample type. For coliforms (96%), *Enterococcus* (87%), and coliphages (65%), the majority of samples tested were positive (Table 1). Only a small proportion of samples were positive for *E. coli* (32%) and *Bacteroidales* (40%). Coliforms and *Enterococcus* had the highest proportion of positive samples regardless of sample type. For all sample types, coliforms had the highest proportion of positive samples, ranging from 93-97% of samples positive.

When stratified by sample type, hand-rinse and produce samples appeared to have the highest proportions of positive samples (Table 1). Soil samples appeared to have the lowest percentage of positive samples with only 20% of samples positive for *E. coli*, 34% positive for coliphages, and 0% positive for *Bacteroidales*. Some indicators were present in higher proportions than others for certain sample types. For example, 100% of hand-rinse samples were positive for *Enterococcus*, but only 66% of soil samples were. Additionally, most sample types, including irrigation water (38%) showed low *E. coli* proportions around (<38%), but 54% of source water samples were positive for *E. coli.*

To assess concentration of indicators on samples, the arithmetic mean was calculated for the $\log_{10}$-transformed variables (Table 1). Concentration data was not available for norovirus. For produce, hand-rinse, and soil samples, *Enterococcus* and coliforms had the highest concentration measures of any indicator. Interestingly, results differed for source and irrigation water. Source water showed that coliforms and coliphages were highest (1.64; 1.25 cfu/100mL), while irrigation water showed that coliforms and *Bacteroidales* were highest (1.62; 2.75 cfu/100mL). The confidence intervals were narrow indicating that accuracy was high for our concentration measures.

To assess relationships between the presence of indicators, logistic models were used to calculate odds ratios and 95% Wald confidence intervals. *E. coli* was the only indicator that showed a significant intra-indicator relationship of positive presence based on another indicator's presence (Table 2). *E. coli* showed a positive relationship with *Enterococcus*, coliforms, and coliphages. If *E. coli* was present on a sample, *Enterococcus* was 2.84 times more likely to be present (p value=0.0012). When *E. coli* was present, coliforms were 24.54 times more likely to be present (p-value=0.0285) and coliphages were 2.34 times more likely to be present (p value=<0.0001). *Bacteroidales* did not show any significant relationships with other indicators (Table 2).

When stratifying by sample type (Table 3), only two of the relationships showed a strong positive correlation (0.6-0.79) between indicators: *Enterococcus* with coliforms for produce samples (rho=0.6251) and *Enterococcus* with coliphages for produce samples (rho=0.6242). Many of the other relationships, though not strong, were significant. The produce and irrigation water samples showed the largest number of significant relationships between indicators. The relationships between indicators on produce samples were all significant at a p-value of 0.05. For

irrigation and source water samples, coliforms had a moderate positive significant (0.40-0.59) relationship with *E. coli* and *Enterococcus* (rho=0.4818; 0.5618). Coliphages and *E. coli* (rho=0.2963) also showed a positive significant relationship, though it was weak. The major difference between irrigation and source water samples was that irrigation water samples showed a moderate positive significant relationship between *Bacteroidales* and coliforms (rho=0.5017) and *Bacteroidales* and *Enterococcus* (rho=0.4453), while no source water samples showed a relationship between *Bacteroidales* and other indicators. Soil samples showed the fewest number of significant relationships and all were weak or very weak (0-0.39). Two of these weak positive associations in soil samples were significant: coliforms and *Enterococcus* (rho=0.3831) and *E. coli* and coliphages (rho=-0.3133).

Assessing the presence of norovirus

To assess presence of norovirus, a subset of 50 samples was tested and proportions of positive samples were calculated. Only six of fifty samples (12%) were positive for norovirus, with five of the six (10%) positive samples being positive for norovirus genotype II (Table 4). The positive samples came from three different farms. Three of the positive samples were collected from a farm that harvested tomatoes: two from hand-rinses and one from source water. Two positive norovirus samples were collected from a farm that harvested melons: one from a source water sample, and one from a melon itself. The melon sample that was positive was also the only sample positive for genotype I. The final positive sample was a source water sample from a jalapeño farm.

Assessing the relationship between indicator and norovirus presence

Logistic models with odds ratios and 95% Wald confidence intervals were run to determine the relationship between indicators and norovirus. There were no significant relationships found,

thus we can conclude that there is no relationship between indicator presence and norovirus presence.

Discussion

The goal of this study was to determine if bacterial and viral indicators can inform the presence of norovirus on grower's hands, produce, and in water of crops grown along the US-Mexico border. Results revealed three significant intra-indicator presence-absence relationships that all included *E. coli*. Another finding was that while 12% of all samples tested for norovirus were positive, 50% of those samples were from source water. Finally, analysis showed that there were no statistically significant relationships between norovirus and indicators.

Intra-indicator Relationships

Analysis showed that there were three significant intra-indicator presence-absence relationships. These relationships were between *E. coli* and *Enterococcus*, *E. coli* and coliphages, and *E. coli* and coliforms. Previous research supports that *E. coli* and coliforms and *E. coli* and *Enterococcus* are strongly correlated in irrigation or surface water samples (44-46). Prior research on the relationship between *E. coli* and coliphages is limited, but one study looking at surface water in an urban catchment found a moderate relationship (47). *E. coli* are a type of coliform (48) that is more telling of fecal contamination than other coliforms (49). Because *E. coli* are a type of coliform, it is expected that these indicators would have a significant relationship. *Enterococcus* and coliphages had significant, yet lower magnitude relationships with *E. coli* than coliforms with *E. coli*. *E. coli, Enterococcus,* and coliphages are all indicative of fecal contamination (49). Because coliphages are *E. coli*-specific phages and all are indicators of fecal contamination, the relationships of *E. coli* with coliphages and with *Enterococcus* are not surprising. One study found coliphages to be correlated with *E. coli* and a better measure of contamination than other indicators (50). Additional explanations for indicator relationships are needed as research is lacking, but research also supports the need for using alternative indicators or new detection methods for indicators (28).

Norovirus Presence

In order to assess norovirus presence, proportions of positive samples were calculated and if positive, the norovirus genotype present was recorded. In our future steps, we plan to sequence all RT-qPCR positive samples. Results showed that half of all samples positive for norovirus were from source water. Three of the six positive samples were source water samples, one from each produce item (tomato, melon, jalapeño). While three source water samples and two hand-rinse samples were positive for norovirus, it is surprising that norovirus was not found on more hand-rinse samples given that infected workers cause a majority of norovirus outbreaks in the U.S. (4). The expectation would be that farmworkers would have similar rates of infection, though there have not been any studies looking at norovirus contamination on farmworker hands. Source water in our sample included ponds, groundwater, and surface water. Often, reclaimed wastewater is used for irrigation or is released back into surface water, which can harbor enteric viruses, such as noroviruses, even after treatment (51). Because wastewater is coming from human and animal sources, it would be difficult for groundwater to become contaminated, which would explain the discrepancy between our positive source water samples and absence of positive groundwater samples. Many studies have tested for norovirus in environmental water samples including wastewater directly, surface water, groundwater, or nearby bodies of water such as ponds and lakes. These studies appear to differ based on what type of water samples were tested. Studies looking at groundwater seem to conclude that norovirus presence is low (52, 53), which supports our results. Meanwhile, studies looking at ponds, rivers, or surface water are varied on whether they concluded norovirus presence was low or high (54-59). Studies looking at wastewater mostly concluded that norovirus presence was high (51, 60-63). In this study, source water included ground and surface water, so it is surprising that half the samples were positive for norovirus. The difference in our results compared to the literature could stem from

different sample locations around the world, climatic factors, land use properties, or differing detection methods.

Relationship between Indicators and Norovirus

Analysis showed no relationship between norovirus presence and indicator organism presence. Studies have found that bacterial indicators have little to no correlation with viruses across a variety of water samples (64-66). Coliphages are thought to be more similar to norovirus but more research is needed (67). Our results are similar to other studies that found no relationship between indicator organisms and norovirus (68-70). A possible explanation for our results is that our sample size was too small leading to low statistical power.

In summary, all significant intra-indicator relationships involved *E. coli*. Norovirus was present on a low proportion of samples, but half of the positive samples were from source water. Additionally, there was no relationship between norovirus presence and indicator presence. Strengths of this research are the wide variety of sample types tested and that both viral and bacterial indicator organisms were measured. A variety of sample types strengthened the study by accounting for different causes of contamination whether environmental or human. Limitations include only looking at three produce items (melons, jalapeños, tomatoes) and the small sample size for norovirus testing. To draw conclusions about produce in general, additional research on a wider variety of produce items would be needed. To improve results regarding norovirus testing, the sample size should be increased to improve statistical power.

Results presented here can inform future research regarding relationships between indicators and detection of norovirus contamination in environmental samples. The outcome of this study does not offer a recommendation for which indicator microbe is best at predicting norovirus contamination. Indicator and norovirus contamination differ widely based on many factors, and so, the appropriate indicator microbe is dependent upon those factors. The results

of this study underscore norovirus tests that are sensitive and specific, as well as cost effective and field-friendly. Overall, future work should focus more on norovirus-specific tests, rather than trying to determine which indicator microbe is most effective at predicting norovirus contamination.

Implications

- *E. coli* shows relationships with many fecal indicators, including *Enterococcus*, coliforms, and coliphages. However, only *E. coli* and *Enterococcus* are widely used to measure fecal contamination, which suggests the need for additional research on indicator relationships and re-evaluation of what indicators are best for measuring contamination.
- Source water accounted for half of the positive norovirus samples in this study while none of the positive samples were from irrigation water. This underscores the importance of determining where norovirus contamination is coming from.
- Half of the norovirus positive samples in our study were from source water samples, yet the literature is conflicting on whether source water has high or low norovirus contamination suggesting that other factors may be involved such as: persistence of norovirus in the environment, ability of the virus to grow in certain samples, or differences in detection ability, temperature, or rainfall and runoff.
- There is no relationship of indicator presence with norovirus presence indicating that current detection methods of using indicator organisms to prevent norovirus contamination may be inadequate for predicting norovirus contamination.
- Bacterial and viral indicators do not appear to be good predictors for the presence of norovirus on grower's hands, produce, and in water of crops grown along the U.S.-Mexico border so alternative detection methods for norovirus contamination in agricultural settings are needed.

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Figures and Tables

Table 1. Proportion of positive samples and mean concentration of log-transformed variables for indicator microbes and norovirus, entire sample and stratified by sample type

Sample Type	Indicator	N	Proportion Positive n/N (%)	N	Concentration Mean ± SE[a,b]	95% Confidence Interval
Produce	Coliforms	239	233/239 (97)	239	5.34 ± 0.13	5.08, 5.60
	E. coli	243	65/243 (27)	243	1.37 ± 0.13	1.11, 1.63
	Enterococcus	243	211/243 (87)	243	5.15 ± 0.16	4.84, 5.45
	Coliphages	181	149/181 (82)	181	2.17 ± 0.14	1.90, 2.45
	Bacteroidales	77	39/77 (51)	77	3.05 ± 0.38	2.30, 3.79
	Norovirus	**17**	**1/17 (6)**	**---**	**---**	**---**
Source water	Coliforms	41	39/41 (95)	41	1.64 ± 0.16	1.32, 1.96
	E. coli	48	26/48 (54)	48	0.13 ± 0.14	-0.16, 0.42
	Enterococcus	45	39/45 (87)	45	0.51 ± 0.13	0.25, 0.76
	Coliphages	26	12/26 (46)	26	1.25 ± 0.36	0.51, 1.98
	Bacteroidales	17	1/17 (6)	17	0.36 ± 0.36	-0.40, 1.12
	Norovirus	**8**	**3/8 (38)**	**---**	**---**	**---**
Irrigation water	Coliforms	71	66/71 (93)	71	1.62 ± 0.15	1.32, 1.93
	E. coli	73	28/73 (38)	73	-0.16 ± 0.10	-0.35, 0.03
	Enterococcus	72	60/72 (83)	72	0.46 ± 0.14	0.19, 0.74
	Coliphages	46	21/46 (46)	46	1.00 ± 0.23	0.54, 1.47
	Bacteroidales	22	10/22 (45)	22	2.75 ± 0.69	1.32, 4.18
	Norovirus	**5**	**0/5 (0)**	**---**	**---**	**---**
Hand-rinse	Coliforms	168	161/168 (96)	168	5.75 ± 0.15	5.46, 6.03
	E. coli	168	62/168 (37)	168	2.41 ± 0.14	2.13, 2.69
	Enterococcus	168	168/168 (100)	168	6.53 ± 0.12	6.30, 6.77
	Coliphages	127	83/127 (65)	127	2.15 ± 0.15	1.85, 2.45
	Bacteroidales	52	17/52 (33)	52	1.82 ± 0.39	1.04, 2.60
	Norovirus	**18**	**2/18 (11)**	**---**	**---**	**---**
Soil	Coliforms	84	80/84 (95)	84	2.52 ± 0.12	2.28, 2.76
	E. coli	85	17/85 (20)	85	-0.03 ± 0.12	-0.27, 0.21
	Enterococcus	85	56/85 (66)	85	1.38 ± 0.10	1.18, 1.59
	Coliphages	65	22/65 (34)	65	-0.39 ± 0.12	-0.62, -0.16
	Bacteroidales	0	0/0 (0)	0	---	---
	Norovirus	**0**	**0/0 (0)**	**---**	**---**	**---**
Entire Sample	Coliforms	622	597/622 (96)	---	---	---
	E. coli	617	198/617 (32)	---	---	---
	Enterococcus	613	534/613 (87)	---	---	---
	Coliphages	459	299/459 (65)	---	---	---
	Bacteroidales	168	67/168 (40)	---	---	---
	Norovirus	**50**	**6/50 (12)**	**---**	**---**	**---**

[a]Concentration measures for norovirus samples are blank because concentration data was not available for these samples.

[b]Concentration measures for the entire sample are blank because units were not comparable.

Table 2. Intra-Indicator Relationship based on Presence for entire sample—Odds Ratio, (95% confidence interval), p-value

	E. coli	***Enterococcus***	**Coliforms**	**Coliphages**	***Bacteroidales***
E. coli	1.00	2.84 (1.51-5.35) 0.0012*	24.54 (1.40-429.99) 0.0285*	2.34 (1.53-3.59) <0.0001*	0.54 (0.29-1.00) 0.0510
Enterococcus	2.84 (1.51-5.34) 0.0012*	1.00	2.45 (0.94-6.37) 0.0673	1.31 (0.77-2.24) 0.3211	1.09 (0.39-3.06) 0.8764
Coliforms	24.51 (1.47-407.45) 0.0257*	2.44 (0.94-6.37) 0.0673	1.00	1.70 (0.56-5.14) 0.3498	1.10 (0.18-6.80) 0.9155
Coliphages	2.34 (1.53-3.59) <0.0001*	1.31 (0.77-2.24) 0.3211	1.70 (0.56-5.14) 0.3497	1.00	0.69 (0.33-1.47) 0.3355
Bacteroidales	0.54 (0.29-1.00) 0.0509	1.09 (0.38-3.13) 0.8794	1.10 (0.18-6.79) 0.9161	0.69 (0.33-1.47) 0.3355	1.00

*Denotes significance based on p-value=0.05 or less and confidence interval

Table 3. Correlation Matrix for Intra-Indicator Relationship based on Concentration for sample type—Spearman correlation

		Coliforms	***E. coli***	***Enterococcus***	**Coliphages**	***Bacteroidales***
Produce	Coliforms	1.00				
	E. coli	0.2574*	1.00			
	Enterococcus	0.6251*	0.4631*	1.00		
	Coliphages	0.4164*	0.3782*	0.6242*	1.00	
	Bacteroidales	0.4290*	0.2852*	0.3291*	0.2440*	1.00
Source water	Coliforms	1.00				
	E. coli	0.5457*	1.00			
	Enterococcus	0.5314*	0.5841*	1.00		
	Coliphages	0.0416	0.4120*	0.3334	1.00	
	Bacteroidales	0.2000	0.4136	-0.0766	---	1.00
Irrigation water	Coliforms	1.00				
	E. coli	0.4818*	1.00			
	Enterococcus	0.5618*	0.5937*	1.00		
	Coliphages	0.2089	0.2963*	0.2211	1.00	
	Bacteroidales	0.5017*	0.1767	0.4453*	0.1428	1.00
Hand rinse	Coliforms	1.00				
	E. coli	0.2412*	1.00			
	Enterococcus	0.5558*	0.3915*	1.00		
	Coliphages	0.0506	0.1586	0.15	1.00	
	Bacteroidales	0.1387	-0.1944	0.0977	0.1533	1.00
Soil	Coliforms	1.00				
	E. coli	0.0821	1.00			
	Enterococcus	0.3831*	-0.0697	1.00		

Coliphages	0.1499	-0.3133*	0.1483	1.00	
Bacteroidales	---	---	---	---	1.00

*Denotes significance when p value=0.05 or less

Table 4. Proportion of Samples positive for Norovirus by sample type and produce type

	Number of Samples	Number (%) positive for Norovirus Genotype		Total Number (%) Positive for Norovirus
Sample Type	**N**	**Genotype I N (%)**	**Genotype II N (%)**	**N (%)**
Hand rinse	18	0/18 (0)	2/18 (11)	2/18 (11)
Irrigation Water	5	0/5 (0)	0/5 (0)	0/5 (0)
Produce	17	1/17 (6)	0/17 (0)	1/17 (6)
Soil	0	---	---	---
Source Water	8	0/8 (0)	3/8 (38)	3/8 (38)
Produce Type				
Jalapeños	14	0/14 (0)	1/14 (7)	1/14 (7)
Melons	15	1/15 (7)	1/15 (7)	2/15 (13)
Tomatoes	19	0/19 (0)	3/19 (16)	3/19 (16)
TOTAL	50	1/50 (2)	5/50 (10)	6/50 (12)

Table 5. Relationship between indicator presence and norovirus presence for entire sample—Odds ratio (95% Confidence Interval), p-value

	Coliforms	***E. coli***	***Enterococcus***	**Coliphages**	***Bacteroidales***
Norovirus	1.60 (0.06-46.74) 0.7842	1.20 (0.24-6.16) 0.8227	1.36 (0.05-37.60) 0.8575	0.93 (0.09-10.10) 0.9531	0.88 (0.07-11.24) 0.9183
Genotype I	0.33 (0.01-12.63) 0.5475	0.38 (0.01-10.46) 0.5656	0.42 (0.01-14.92) 0.6322	1.00 (0.03-30.23) 1.000	5.82 (0.19-179.63) 0.3139
Genotype II	1.32 (0.05-39.16) 0.8724	1.76 (0.30-10.27) 0.5303	1.03 (0.04-29.56) 0.9877	0.60 (0.05-7.41) 0.6904	0.03 (0.01-8.29) 0.4812

Appendix A: IRB Approval

EMORY UNIVERSITY

Institutional Review Board

TO: Juan Leon, PhD
Principal Investigator
*SPH: Global Health

DATE: July 10, 2018

RE: **Continuing Review Expedited Approval**
CR8_IRB00035460

IRB00035460
Identification and Control of Microbiological Hazards in Imported Fresh Fruits and Vegetables: A Field Epidemiological and Intervention Study in Northern Mexico

Thank you for submitting a renewal application for this protocol. The Emory IRB reviewed it by the expedited process on **07/09/2018**, per 45 CFR 46.110, the Federal Register expeditable category [F7], and/or 21 CFR 56.110. This reapproval is effective from **07/09/2018** through **07/08/2019**. Thereafter, continuation of human subjects research activities requires the submission of another renewal application, which must be reviewed and approved by the IRB prior to the expiration date noted above. Please note carefully the following items with respect to this reapproval:

- Consent Documents
  - consentimiento_enjuaguemanos_11.22.2017_CLEAN.docx
  - Informacion-Encuesta Manipulador 23 MAR 2011.doc
  - Informacion-Encuesta-Productor-Manager 23 MAR 2011.docx
  - Oral Script for Written Consent_FarmManagerSurvey_Spanish_4.26.2011.doc
  - Oral Script for Written Consent_FarmManagerSurvey_ver4.26.2011_CLEAN.doc
  - OralScript_Hand Rinsing_ver11.22.2017_CLEAN.docx
- Protocol Document
  - CGProtocol_11.22.17CLEAN.docx

Any reportable events (e.g., unanticipated problems involving risk to subjects or others, noncompliance, breaches of confidentiality, HIPAA violations, protocol

deviations) must be reported to the IRB according to our Policies & Procedures at www.irb.emory.edu, immediately, promptly, or periodically. Be sure to check the reporting guidance and contact us if you have questions. Terms and conditions of sponsors, if any, also apply to reporting.

Before implementing any change to this protocol (including but not limited to sample size, informed consent, and study design), you must submit an amendment request and secure IRB approval.

In future correspondence about this matter, please refer to the IRB file ID, name of the Principal Investigator, and study title. Thank you.

Sincerely,

Heather Yates
Analyst Assistant
This letter has been digitally signed

CC: Prince-Guerra Jessica *SPH: Global Health

Names of other Emory Study Staff not listed above. If name does appear in menu, the person probably does not yet have an eIRB account. For more information about obtaining an eIRB account,

	Last	First	Dept	Type
View	Nace	Molly	*SPH: Global Health	Data entry - data analysis
View	Sobolik	Julia	*SPH: Global Health	Research Fellow (after July 2015: if acting as investigator, list in Co-Investigator group instead; i.e. if doing protocol-driven procedures, consenting, drug/device orders...)
View	Wickson	Alexandra	*SPH: Global Health	Data entry - data analysis